Supplementary Information

Inhibition of the 4Fe-4S Proteins IspG and IspH: an EPR, ENDOR and HYSCORE Investigation

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Synthetic aspects.

General methods. All reagents used in chemical synthesis were purchased from Aldrich (Milwaukee, WI). The structures of all compounds investigated were confirmed by using $^1$H and $^{31}$P NMR spectroscopy at 400 MHz or 500 MHz on Varian (Palo Alto, CA) Unity spectrometers. Cellulose TLC plates were visualized by using iodine or a sulfosalicylic acid-ferric chloride stain.

Synthesis of $[3^{-13}$C]-But-3-ynyl diphosphate ($[3^{-13}$C]-5). The synthesis of ($[3^{-13}$C]-5 was carried out as shown in the following Scheme.

\[
\begin{align*}
\text{Cl} & \quad \text{OH} \\
\text{H} & \quad \text{OTBDPS} \\
\text{13} & \quad \text{O}
\end{align*}
\]

Reagents and conditions: a) K$^{13}$CN, EtOH-H$_2$O, 80 °C, 12h; b) TBDPSCl, imidazole, CH$_2$Cl$_2$, 0 °C ; c) DIBAL-H, -40 °C, 4h; d) CBr$_4$, PPh$_3$, Zn, CH$_2$Cl$_2$, r. t., 48h; e) i. n-BuLi, THF, -78 °C, 1h; ii. H$_2$O; f) TBAF, THF, r. t.; g) MsCl, Et$_3$N, CH$_2$Cl$_2$, 0 °C ; h) (n-Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, 0 °C – r. t., 48 h.

$[1^{-13}$C]-3-Hydroxypropionitrile (11)

$[1^{-13}$C]-3-Hydroxypropionitrile (11) was synthesized according to a literature method (1). A flask was charged with 530 mg of K$^{13}$CN (8 mmol) and 5 mL of water was added. A 10% solution of NaI in ethanol (15 mL) was added, followed by 1.6 g (20 mmol) of 2-chloroethanol. The reaction was heated at 80 °C for 12 h then cooled to room temperature. A negative cyanide test, shown by no change in color upon addition of 1 drop of reaction mixture to 0.5 mL of a 1.0 M solution of p-nitrobenzaldehyde in DMSO, demonstrated the consumption of all cyanide. The solvent was removed in vacuo and the residue purified by flash silica chromatography (hexane : EtOAc = 1 : 1) yielding 430 mg (75 %) of $[1^{-13}$C]-3-Hydroxypropionitrile (11).
$^{13}$C]-3-hydroxypropionitrile as a clear oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.89 (dq, $J = 0.9$ Hz, 6.3 Hz, 2H), 2.64 - 2.58 (m, 2H), 2.10 - 1.80 (m, 1H).

[1-$^{13}$C]- 3-($t$-butyldiphenylsilyloxy)propionitrile (12)

A solution of 355 mg (5 mmol) of 11 in 20 mL of CH$_2$Cl$_2$ was cooled to 0 °C. 340 mg (5 mmol) of imidazole was added and the mixture stirred for 10 min before addition of 1.37 g (5 mmol) $t$-butylchlorodiphenylsilane. The reaction mixture was then slowly allowed to warm to room temperature, over 12 h. Saturated aqueous ammonium chloride (10 mL) was added and the layers separated. The aqueous layer was extracted with CH$_2$Cl$_2$, the combined organic layers concentrated in vacuo, and the residue purified by flash silica chromatography (hexane : EtOAc = 6 : 1) to give 1.41 g (91 %) of 12 as a colorless solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.66 - 7.64 (m, 4H), 7.42 - 7.24 (m, 6H), 3.82 (q, $J = 6.0$ Hz, 2H), 2.56 - 2.50 (m, 2H), 1.05 (s, 9H).

[1-$^{13}$C]- 3-($t$-butyldiphenylsilyloxy)propionaldehyde (13)

A stirred solution of 12 (920 mg, 3 mmol) in dichloromethane (3.0 mL) was cooled to - 40 °C, and 3.0 mL of diisobutylaluminum hydride (1.0 M in hexanes) added dropwise. The reaction mixture was stirred at - 40 °C for 4 hrs and then quenched with aqueous acetic acid. The resulting insoluble materials were filtered through Celite and washed with acetone. The combined organic layers were concentrated in vacuo and the residue purified by flash silica chromatography (hexane : EtOAc = 6 : 1) to give 0.51 g (55 %) of 13 as a colorless solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.80 (dt, $^1J_{H,C} = 172.8$ Hz, $J = 2.0$ Hz, 1H), 7.66 - 7.63 (m, 4H), 7.41 - 7.36 (m, 6H), 4.01 (q, $J = 6.0$ Hz, 2H), 2.62 - 2.56 (m, 2H), 1.06 (s, 9H).

[3-$^{13}$C]-$t$-Butyl-(4, 4-dibromo-but-3-en-1-yloxy)diphenylsilane (14)

[3-$^{13}$C]-$t$-Butyl-(4, 4-dibromo-but-3-en-1-yloxy)diphenylsilane (14) was synthesized according to a literature method (2). A solution of 312 mg (1 mmol) of 13 in 1.5 mL of dry CH$_2$Cl$_2$ was treated at room temperature with 664 mg (2 mmol) of CBr$_4$ and 132 mg (2 mmol) of zinc dust, followed by 262 mg (1 mmol) of PPh$_3$, in small portions. The resulting mixture was stirred at room temperature for 48 h, diluted with 30 mL of hexanes, filtered, and washed with ether. The combined organic layers were concentrated in vacuo and the residue purified by
flash silica chromatography (hexane : EtOAc = 25 : 1) to yield 360 mg (77 \%) of 14 as a colorless oil. 

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.66 - 7.63 (m, 4H), 7.44 - 7.35 (m, 6H), 6.45 (dt, $^1J_{H,C} = 161.2$ Hz, $J = 6.8$ Hz, 1H), 3.71 - 3.67 (m, 2H), 2.36 - 2.29 (m, 2H), 1.04 (s, 9H).

$[3-^{13}C]$-t-Butyl-(2-methylpent-3-ynyloxy)diphenylsilane (15)

To a solution of 188 mg (0.4 mmol) of 14 in 4.0 mL of dry THF was added dropwise 0.30 mL of n-BuLi (1.6 M solution in hexanes) at -78 °C. The reaction mixture was stirred at -78 °C for 1 h and then quenched with H$_2$O and extracted with ether. The combined organic layers were dried over MgSO$_4$, concentrated in vacuo and purified by flash silica chromatography (hexane : EtOAc = 25 : 1) to yield 116 mg (94\%) of 15 as a colorless oil. $^1$H NMR (500 MHz, CDCl$_3$): 7.69 - 7.66 (m, 4H), 7.44 - 7.37 (m, 6H), 3.80 - 3.77 (m, 2H), 2.48 - 2.43 (m, 2H), 1.95 (dt, $^2J_{H,C} = 49.5$ Hz, $J = 2.5$ Hz, 1H), 1.05 (s, 9H).

$[3-^{13}C]$-But-3-yn-1-ol (16)

15 (100 mg, 0.32 mmol) in 0.30 mL THF was added slowly at room temperature to 0.30 mL of tetrabutylammonium fluoride (TBAF, 1.0 M solution in THF). After 30 minutes, the reaction mixture was quenched with water. The aqueous layer was extracted twice with EtOAc. The resulting organic layers were filtered, concentrated and purified by flash silica chromatography (hexane : EtOAc = 1 : 2) to provide 16 as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.78 - 3.71 (m, 2H), 2.51-2.46 (m, 2H), 2.06 (dt, $^2J_{H,C} = 49.5$ Hz, $J = 2.5$ Hz, 1H).

$[3-^{13}C]$-But-3-ynyl methanesulfonate (17)

$[3-^{13}C]$-But-3-yn-1-ol (16) 14.2 mg (0.2 mmol) was dissolved in 0.5 mL CH$_2$Cl$_2$ and cooled to 0 °C. 23 mg (0.2 mmol) of methanesulfonyl chloride was added dropwise at 0 °C followed by the addition of 20 mg (0.2 mmol) of Et$_3$N. The reaction mixture was stirred for 30 minutes at 0°C and then quenched with water. The aqueous layer was extracted once with CH$_2$Cl$_2$, the combined organic layers concentrated under reduced pressure, and then purified by flash silica chromatography (hexane : EtOAc = 1 : 2) to provide 23 mg (77 \%) of 17 as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.32 - 4.27 (m, 2H), 3.04 (s, 3H), 2.68 - 2.63 (m, 2H), 2.08 (dt, $^2J_{H,C} = 50.0$Hz, $J = 2.4$ Hz, 1H).

$[3-^{13}C]$-But-3-ynyl diphosphate ([3–$^{13}$C]-5)
[3-^{13}C]-But-3-ynyl diphosphate ([3-^{13}C]-5) was synthesized according to a literature method (3). [3-^{13}C]-but-3-ynyl methanesulfonate (17) (15 mg, 0.1 mmol) in dry CH$_3$CN (0.3 mL) was added to a stirred solution of 0.27 g (0.3 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in dry CH$_3$CN (0.5 mL) at 0 °C. The reaction mixture was slowly allowed to warm to room temperature over 24 h, then solvent was removed under reduced pressure. The residue was dissolved in 0.5 mL of cation-exchange buffer (49:1(v/v) 25 mM NH$_4$HCO$_3$/2-propanol) and passed over 90 mequiv of Dowex AG50W-X8 (100-200 mesh, ammonium form) cation-exchange resin, pre-equilibrated with two column volumes of the same buffer. The product was eluted with two column volumes of the same buffer, flash frozen, and lyophilized. The resulting powder was dissolved in 0.5 mL 50 mM NH$_4$HCO$_3$, 2-propanol/CH$_3$CN (1:1 (v/v), 1 mL) added, and the mixture mixed on a vortex mixer, then centrifuged for 5 min at 805 g. The supernatant was decanted. This procedure was repeated three times and the supernatants were combined. After removal of the solvent and lyophilization, a white solid was obtained. Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH$_3$CN/50 mM NH$_4$HCO$_3$) yielded 9 mg (30%) of [3-^{13}C]-5 as a colorless solid. $^1$H NMR (500 MHz, D$_2$O): $\delta$ 3.93 - 3.85 (m, 2H), 2.45 - 2.40 (m, 2H), 2.23 (dt, $^2J_{H,C}$ = 50.5 Hz, $^1J_{H,H}$ = 2.5 Hz, 1H). $^{31}$P NMR (200 MHz, D$_2$O): $\delta$ -8.55 (m), -10.0 (m).

**Synthesis of [4-^{13}C]-But-3-ynyl diphosphate ([4-^{13}C]-5)**

[4-^{13}C]-But-3-ynyl diphosphate ([3-^{13}C]-5) was prepared in a similar manner to that used to prepare [3-^{13}C]-5, as illustrated in the following scheme:

Reagents and conditions: a) $^{13}$CB$_{4}$, PPh$_3$, Zn, CH$_2$Cl$_2$, r. t., 48h; b) i. $n$-BuLi, THF, -78 °C, 1h; ii. H$_2$O; c) TBAF, THF, r. t.; d) MsCl, Et$_3$N, CH$_2$Cl$_2$, 0 °C; e) ($n$-Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, 0 °C – r. t., 48 h.

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3-(t-Butyldiphenylsilyloxy)propionaldehyde was synthesized following the procedure for [1-\textsuperscript{13}C]-3-(t-butyldiphenylsilyloxy) propionaldehyde (13) described above. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \( \delta \) 9.82 (t, \( J = 2.0 \) Hz, 1H), 7.70 – 7.66 (m, 4H), 7.45 – 7.38 (m, 6H), 4.03 (t, \( J = 6.0 \) Hz, 2H), 2.61 (td, \( J = 6.0, 2.4 \) Hz, 2H), 1.06 (s, 9H).

\textbf{[4-\textsuperscript{13}C]-t-Butyl-(4, 4-dibromo-but-3-enyloxy)diphenylsilane (18)}

A solution of 114 mg (0.37 mmol) of 3-(t-butyldiphenylsilyloxy)propanal in 0.5 mL of dry CH\textsubscript{2}Cl\textsubscript{2} was treated at room temperature with 100 mg (0.3 mmol) of \textsuperscript{13}CBr\textsubscript{4} and 40 mg (0.6 mmol) of zinc dust, followed by 95 mg (0.36 mmol) of PPh\textsubscript{3}, in portions. The resulting mixture was stirred at room temperature for 48 h, diluted with 10 mL of hexanes, filtered, and washed with ether. The combined organic layers were concentrated \textit{in vacuo}, and the residue purified by flash silica chromatography (hexane : EtOAc = 25 : 1) to yield 147 mg (85 \%) of 18 as a colorless oil. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}):
\[ \delta 7.68 - 7.65 \text{ (m, 4H)}, 7.43 - 7.36 \text{ (m, 6H)}, 6.48 \text{ (dt, } J = 6.8 \text{ Hz, } J = 1.2 \text{ Hz, 1H)}, 3.71 \text{ (t, } J = 6.0 \text{ Hz, 2H)}, 2.38 - 2.30 \text{ (m, 2H)}, 1.06 \text{ (s, 9H)}.\]

\textbf{[4-\textsuperscript{13}C]-t-Butyl-(2-methylpent-3-ynyloxy)diphenylsilane (19)}

The procedure described for [3-\textsuperscript{13}C]-t-butyl-(2-methylpent-3-ynyloxy) diphenylsilane (15) was used for the synthesis of [4-\textsuperscript{13}C]-t-butyl-(2-methylpent-3-ynyloxy)diphenylsilane (19). A solution of 94 mg (0.2 mmol) of 18 in 2.0 mL of dry THF was treated at -78 °C with 0.15 mL (0.24 mmol) of \( n \)-BuLi (1.6 M solution in hexanes). The reaction mixture was stirred at -78 °C for 1 h and quenched with H\textsubscript{2}O and then extracted with ether. The combined organic layers were dried over MgSO\textsubscript{4}, concentrated \textit{in vacuo}, and purified by flash silica chromatography (hexane : EtOAc = 25 : 1) to yield 58 mg (94\%) of 19 as a colorless oil. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}):
\[ \delta 7.67 - 7.65 \text{ (m, 4H)}, 7.42 - 7.32 \text{ (m, 6H)}, 6.48 \text{ (dt, } J = 6.8 \text{ Hz, } J = 1.2 \text{ Hz, 1H)}, 3.76 \text{ (t, } J = 6.8 \text{ Hz, 2H)}, 2.46 - 2.40 \text{ (m, 2H)}, 1.93 \text{ (dt, } J_{H, C} = 248.4 \text{ Hz, } J = 2.8 \text{ Hz, 1H)}, 1.04 \text{ (s, 9H)}.\]

\textbf{[4-\textsuperscript{13}C]-But-3-yn-1-ol (20)}

[4-\textsuperscript{13}C]-t-Butyl-(2-methylpent-3-ynyloxy)diphenylsilane (19) (100 mg, 0.32 mmol) in 0.30 mL THF was added slowly at room temperature to 0.30 mL of TBAF (1.0 M solution in THF). After an additional 30 minutes, the reaction mixture was
quenched with water. The aqueous layer was extracted twice with EtOAc. The resulting organic layer was filtered, concentrated, and purified by flash silica chromatography (hexane : EtOAc = 1 : 2) to provide a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.75 (t, $J = 6.5$ Hz, 2H), 2.50 - 2.45 (m, 2H), 2.05 (dt, $^1J_{H,C} = 249.0$ Hz, $J = 2.5$ Hz, 1H).

**[4-^{13}C]-But-3-ynyl methanesulfonate (21)**

[4-^{13}C]-But-3-yn-1-ol (20) 14 mg (0.2 mmol) was dissolved in 0.5 mL CH$_2$Cl$_2$ and cooled to 0 °C. Then, 23 mg (0.2 mmol) of methanesulfonyl chloride was added dropwise at 0 °C, followed by the addition of 20 mg (0.2 mmol) of Et$_3$N. The reaction mixture was stirred for 30 minutes at 0°C and then quenched with water. The aqueous layer was extracted once with CH$_2$Cl$_2$, the combined organic layers concentrated under reduced pressure, then purified by flash silica chromatography (hexane : EtOAc = 1 : 2) to provide 23 mg (77 %) of 21 as a colorless oil. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 4.32 (t, $J = 6.5$ Hz, 2H), 3.07(s, 3H), 2.66 - 2.70 (m, 2H), 2.08 (dt, $^1J_{H,C} = 250.5$ Hz, $J = 2.5$ Hz, 1H).

**[4-^{13}C]-But-3-ynyl diphosphate ([4-^{13}C]-5)**

Following the procedure described for [3-^{13}C]-but-3-ynyl diphosphate ([3 – ^{13}C] - 5), [4-^{13}C]-but-3-ynyl methanesulfonate (21) (15 mg, 0.1 mmol) in dry CH$_3$CN (0.3 mL) was added to a stirred solution of 0.27 g (0.3 mmol) tris(tetra-n-butylammonium) hydrogen diphosphate in dry CH$_3$CN (0.5 mL), at 0 °C. The reaction mixture was allowed to warm to room temperature over 48 h. Flash chromatography on a cellulose column (2:1:1 (v/v/v) 2-propanol/CH$_3$CN/50 mM NH$_4$HCO$_3$) yielded 9 mg (30%) of [4-^{13}C]-5 as a colorless solid. $^1$H NMR (500 MHz, D$_2$O): $\delta$ 5.51 - 5.45 (m, 1H), 3.90 - 3.85 (m, 2H), 2.44 - 2.40 (m, 2H), 2.22 (dt, $^1J_{H,C} = 250$ Hz, $J = 3.0$ Hz, 1H); $^{31}$P NMR (200 MHz, D$_2$O): $\delta$ - 8.55 (d, $J = 19.6$ Hz), - 10.0 (d, $J = 19.6$ Hz).

**[U-^{13}C]-E-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate ([U-^{13}C]-2)**

Preparation of [U-^{13}C]-2 was described previously (4).

**E. coli IspG protein expression and purification.** Protein was expressed and purified basically as described in Ref. (4). BL-21(DE3) cells
overexpressing *E. coli* IspG (encoded in plasmid pASK- IBA5+) and isc proteins (encoded in plasmid pDB1282) were grown in LB media supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin at 37 °C, until the OD$_{600}$ reached 0.3. Cells were then induced with 0.5 g/L D-arabinose to initiate overexpression of the isc proteins. Cysteine (0.5 mM) and FeCl$_3$ (0.1 mM) were added, and the cells grown until the OD$_{600}$ reached 0.6. At this point, 400 μg/L anhydrotetracycline was added, to induce overexpression of *E. coli* IspG. Cells were grown at 21 °C for a further 24 hours, then harvested by centrifugation (14,334 g, 8 min, 4 °C). The cell pellets were kept at -80 °C until further use.

All purification steps were carried out in a Coy vinyl anaerobic chamber (Coy Laboratories, Grass Lake, MI) with an oxygen level < 2 ppm, and all buffers were degassed by using a Schlenk line. Cell pellets were resuspended in 100 mM Tris-HCl, 150 mM NaCl buffer (pH 8.0). Lysozyme, benzonase nuclease (EMD Chemicals, San Diego, CA) and phenylmethanesulfonyl fluoride were added, and the slurry stirred for 1.5 hours at 10 °C, followed by sonication (Fisher Scientific Sonic Dismembrator, Model 500) with 4 pulses, each of 7 sec duration at 35% power. The cell lysate was then centrifuged at 15,550 g at 10 °C for 30 min. The supernatant was purified by using Strep-tactin chromatography (8). Fractions having a brown color were collected and desalted in pH 8.0 buffer containing 100 mM Tris-HCl and 150 mM NaCl.

*A. thaliana* IspG protein expression and purification. The pQE-31 plasmid containing an N-terminal truncated (signal- and transit-peptide cleaved) *At*IspG gene was co-transformed with the Kan$^R$ pDB1281 plasmid in *Escherichia coli* BL21 (DE3) RIPL competent cells (Agilent Technologies) (9). *E. coli* cells were grown aerobically in 100 mL sterile LB (50 μg/mL ampicillin, 25 μg/mL kanamycin, 11 μg/mL chloramphenicol) for 12 h at 37 °C, shaking at 250 rpm. A 50 mL inoculum was transferred to 1.5 L of sterile LB broth (50 μg/mL ampicillin, 25 μg/mL kanamycin, 11 μg/mL chloramphenicol) in 4 L flasks and shaken at 185 rpm at 37 °C. At an OD$_{600}$ of ~ 0.2, 0.5 g/L D-arabinose (to induce expression of pDB1281 genes), 200 μM FeCl$_3$, and 1 mM L-cysteine were added to each 1.5 L LB flask, then the cells were grown at 37 °C with shaking at 185 rpm. At

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an OD$_{600}$ of ~ 1.0, protein expression was induced by adding 1 mM IPTG to each flask. The cultures were then grown at 28 °C for 12-14 h, shaking at 160 rpm. Cells were harvested by centrifugation at 14,334 g using an SLC-3000 rotor and a Sorvall Evolution centrifuge. Cell pellets were stored at -80 °C for later purification. All steps for anaerobic purification of AtIspG were performed in a Coy glove box at 4 °C. The cell pellet was lysed with 10 mg/mL lysozyme (chicken egg white), 62 U/mL benzonase nuclease (purity > 90%), and 1 tablet/50mL protease inhibitor cocktail, in a slowly stirred binding buffer (5 mM imidazole, 200 mM Hepes, 100 mM KCl, pH 7.6). The cell lysate was sonicated for 90 s with on/off intervals of 10 s / 20 s, respectively and then centrifuged at 2,056 g in an F-34-6-38 rotor in a table-top Eppendorf 5804 centrifuge for 90 min (transferring the supernatant to new centrifuge tubes after 45 min to remove most of the precipitate). The supernatant was filtered using a syringe-driven 0.45 μm filter, loaded onto a nickel affinity column, and purified as follows. First, bound protein was washed with 100-200 mL 50 mM imidazole (200 mM Hepes, 100 mM KCl, pH 7.6). IspG was then eluted with 250 mM imidazole (200 mM Hepes, 100 mM KCl, pH 7.6) and concentrated using 10K-50K centrifugal filters. The concentrated protein was then loaded onto a PD-10 desalting column, eluted with final buffer (200 mM Hepes, 100 mM KCl, pH 7.6), and concentrated by centrifugation. All purified protein batches were run on SDSPAGE gels to verify purity. MALDI-TOF mass spectra of AtIspG exhibited a major peak at 82,247 Da (expected 82,157). A BioRad protein concentration assay was used to determine final protein concentration. Protein was stored at -80 °C.

**P. aeruginosa IspG protein expression and purification.** The pET-26b plasmid containing the *Pa*IspG gene was co-transformed with the Amp$^R$ pDB1282 plasmid in *E. coli* BL21 (DE3) pLysS competent cells (Novagen). Inoculated *E. coli* were grown aerobically in 100 mL LB (with 50 μg/mL ampicillin, 25 μg/mL kanamycin) for 12 h at 37 °C, shaking at 250 rpm. A 50 mL inoculum was transferred to 1 L of sterile LB broth (50 μg/mL ampicillin, 25 μg/mL kanamycin) in 4 L flasks and shaken at 185 rpm at 37 °C. At an OD$_{600}$ of ~ 0.2, 0.5 g/L D-arabinose (to induce expression of pDB1282 genes), 200 μM FeCl$_3$, and 1 mM L-
cysteine were added to each 1 L LB flask, then the cells were grown at 37 °C with shaking at 185 rpm. At an OD$_{600}$ of ~ 1.0, 1 mM IPTG was added to each flask to induce protein expression. The cultures were grown at 28 °C for 14-18 h, shaking at 160 rpm. Cells were harvested by centrifugation at 14,334 g using an SLC-3000 rotor and a Sorvall Evolution centrifuge. Cell pellets were stored at -80 °C for later purification. All steps for anaerobic purification of PaIspG were similar to those used for the AtIspG anaerobic purification described above.

**A. aeolicus IspH protein expression and purification.** A. aeolicus IspH was expressed and purified basically as described in Ref. (3). BL-21(DE3) cells (Invitrogen) expressing IspH from A. aeolicus were grown in LB media supplemented with 150 mg/mL ampicillin at 37 °C until the OD$_{600}$ reached ~0.6. Cells were then induced with 200 µg/L anhydrotetracycline and grown at 20 °C for 15 h. Cells were harvested by centrifugation (14,334 g, 20 min, 4 °C) and kept at -80 °C until further use. Purification was then carried out in an anaerobic chamber in a 4 °C cold room. Cell pellets were re-suspended and lysed by sonication, then centrifuged at 15,550 g at 4 °C for 20 min. The supernatant was applied to a HisPur Ni-NTA spin column (Pierce) equilibrated with a pH 8.0 buffer containing 50 mM Tris · HCl and 150 mM NaCl. After washing with 6 mL of 25 mM imidazole-containing buffer 3 times, protein was eluted with buffer containing 250 mM imidazole. Dark-brown fractions were collected, pooled, concentrated and then desalted on a PD-10 column (GE Healthcare), to remove imidazole. Because AaIspH does not incorporate iron-sulfur clusters particularly well, even when purified in a glove box, cluster reconstitution was employed. To do this, protein was transferred to a room-temperature Coy vinyl anaerobic chamber, diluted to 1 – 2 mM, and incubated with 20 mM DTT for 1 hour. Then, 0.5 mM FeCl$_3$ and 0.5 mM Na$_2$S were slowly added to the protein solution with gentle stirring (~ 100 rpm), followed by incubation for 6 – 8 hours. These additions and incubations of FeCl$_3$ and Na$_2$S were repeated five times. Care was taken that iron sulfide and protein precipitation were kept to a minimum. The protein solution was then centrifuged at 15,550 g for 20 minutes, the supernatant concentrated,
desalted again (to remove excess metal and sulfide ions), and the purified, reconstituted protein stored in the anaerobic chamber at 4°C.

\[ ^{15} \text{N-labeled } A. \text{aeolicus IspH expression and purification.} \] To express \(^{15}\)N-labeled protein, the above protocol for \( Aa \)IspH was modified by substituting LB medium with M9 minimal medium (minus \(^{14}\)N nitrogen source) supplemented with 4 g/L glucose, 1 g/L \([^{15} \text{N}] \text{-(NH}_4\text{)}_2\text{SO}_4\) (Cambridge Isotope Labs), 1% (v/v) MEM vitamin solution (Sigma-Aldrich), 0.5% (v/v) \(^{15}\)N-labeled BioExpress cell growth medium (Cambridge Isotope Labs), 0.2 mM trisodium citrate and 0.1 mM FeCl\(_3\). Protein expression was induced at 25 °C when the OD\(_{600}\) reached ~ 1.4, and cells were harvested 20 hours after. \(^{15}\)N-labeled \( Aa \)IspH protein was then purified and reconstituted as described above.

\[ E. \text{coli IspH protein expression and purification.} \] BL21(DE3) (Invitrogen) cells harboring an \( E. \) coli IspH construct were grown in LB media at 37°C until the OD\(_{600}\) reached ~0.6. Induction was performed with 200 ng/mL anhydrotetracycline at 20 °C for 15 h. Cells were harvested by centrifugation at 14,334 g for 8 min and stored at -80 °C. Cell pellets were moved into a cold-room glove box as with \( Aa \)IspH, then resuspended and lysed by sonication, and the lysate centrifuged at 15,550 g for 30 min. The supernatant was collected and loaded onto an IBA Strep-tag column equilibrated with buffer W (100 mM Tris · HCl, 150 mM NaCl, pH 8.0). After washing with buffer W, protein was eluted using buffer E (buffer W containing 2.5 mM desthiobiotin). Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris · HCl, 150 mM NaCl, 5% glycerol, and 1 mM DTT, twice. The purified protein was flash-frozen in liquid nitrogen and stored at -80 °C.

\[ \text{Plasmodium falciparum IspH protein expression and purification.} \] A pASK-IBA3plus (Amp\(^{R}\)) plasmid containing a truncated \( Pf \)IspH gene was co-transformed with the \( isc \) operon containing Kan\(^{R}\) pDB1281 plasmid into BL21 (DE3) pLysS (Novagen) competent cells. The expression and purification
procedure followed that for \textit{P. aeruginosa} IspG (see above), except that at OD\textsubscript{600} ~ 0.6-1.0, protein expression was induced by adding 200 μg/L anhydrotetracycline.

\textbf{Thermus thermophilus IspG D87A mutagenesis, protein expression, and purification.} The \textit{TtIspG}-D87A mutant has been described previously, Ref. (5).

\textbf{Enzyme inhibition assays.} All assays were performed inside a Coy vinyl anaerobic chamber with an oxygen level < 2 ppm. 0.3 μM IspG was added to 0.8 mM dithionite (DT) and 4.0 mM methyl viologen (MV) in 0.2 M HEPES, 0.1 M KCl, pH 7.6 buffer and incubated at room temperature, ~25 °C, for 10 min. 140 μM MEcPP was then added to bring the total volume to 200 μL. Initial velocity measurements of the oxidation of methyl viologen were made at 732 nm using a 96-well plate reader (a Molecular Devices SpectraMax Plus 384 Spectrophotometer). Blanks, composed of MEcPP + DT + MV, were run concurrently with each assay, as were positive controls of IspG + MEcPP + DT + MV. The final reaction velocities were baseline corrected, according to the blanks, and normalized to the positive controls. Data were fit to a standard Hill Equation in Matlab (version 2011b, the MathWorks Inc., Natick, MA) and Prism (version 5.0, GraphPad Software, La Jolla, CA) with the final plots made in Prism. The error bars represent the standard deviations; the Hill coefficients varied from 0.98-1.6. In order to make comparisons between all of the IspGs tested, a uniform enzyme concentration of 0.3 μM was used.

\textbf{CW-EPR/ENDOR/HYSCORE Sample preparation.} All samples were prepared inside a Coy Vinyl Anaerobic Chamber with an oxygen level < 2 ppm. Samples were typically 0.2-1.6 mM in IspG, and glycerol was added as a glassing agent to 20% (v/v). 20-80 equivalents of sodium dithionite were added as a reducing agent, and ligands were added to 15-20 equivalents.
**CW-EPR/ENDOR/HYSCORE Simulations.** Simulations were performed in Matlab 7.12.0 (R2011a) with EasySpin 4.0.0 (and 4.5.0) (6). Initial g tensors were interactively determined and used for subsequent refinements with EasySpin’s least squares fitting implementation. ENDOR/HYSCORE simulations were performed with EasySpin’s saffron function (7). A 0.5 MHz uncertainty in hyperfine tensor assignments to HYSCORE data was determined using RMSD and normalized cross-correlation metrics between corresponding data and simulations. The spin-projection coefficient previously reported for aconitase was taken to vary from 0.86 ($K_{\text{min}}$) – 1.78 ($K_{\text{max}}$) (8).
Table S1. EPR $g$-values, $g_{iso}$, and $\Delta g$ for IspG and IspH.

<table>
<thead>
<tr>
<th>Enzyme + Ligand</th>
<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{iso}^a$</th>
<th>$\Delta g^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaIspH reduced</td>
<td>2.041</td>
<td>1.919</td>
<td>1.849</td>
<td>1.936</td>
<td>0.191</td>
</tr>
<tr>
<td>AaIspH + 7</td>
<td>2.134</td>
<td>2.006</td>
<td>1.973</td>
<td>2.038</td>
<td>0.161</td>
</tr>
<tr>
<td>EcIspH reduced</td>
<td>2.037</td>
<td>1.924</td>
<td>1.921</td>
<td>1.960</td>
<td>0.116</td>
</tr>
<tr>
<td>EcIspH + 7</td>
<td>2.138</td>
<td>2.005</td>
<td>1.978</td>
<td>2.040</td>
<td>0.160</td>
</tr>
<tr>
<td>PfIspH + 7</td>
<td>2.136</td>
<td>2.008</td>
<td>1.982</td>
<td>2.042</td>
<td>0.154</td>
</tr>
<tr>
<td>EcIspH E126Q + 2</td>
<td>2.132</td>
<td>2.003</td>
<td>1.972</td>
<td>2.036</td>
<td>0.160</td>
</tr>
<tr>
<td>AaIspH E126A + 2</td>
<td>2.124</td>
<td>1.999</td>
<td>1.958</td>
<td>2.027</td>
<td>0.166</td>
</tr>
<tr>
<td>EcIspG + 8</td>
<td>2.100</td>
<td>2.010</td>
<td>1.975</td>
<td>2.028</td>
<td>0.125</td>
</tr>
<tr>
<td>PaIspG + 8</td>
<td>2.105</td>
<td>2.009</td>
<td>1.972</td>
<td>2.029</td>
<td>0.133</td>
</tr>
<tr>
<td>AtIspG + 8</td>
<td>2.096</td>
<td>2.009</td>
<td>1.974</td>
<td>2.026</td>
<td>0.122</td>
</tr>
<tr>
<td>EcIspG + 2</td>
<td>2.091</td>
<td>2.010</td>
<td>1.976</td>
<td>2.026</td>
<td>0.115</td>
</tr>
<tr>
<td>PaIspG + 2</td>
<td>2.097</td>
<td>2.009</td>
<td>1.978</td>
<td>2.027</td>
<td>0.124</td>
</tr>
<tr>
<td>AtIspG + 2</td>
<td>2.077</td>
<td>2.006</td>
<td>1.974</td>
<td>2.019</td>
<td>0.103</td>
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<tr>
<td>TtIspG D87A + 2</td>
<td>2.098</td>
<td>2.012</td>
<td>1.978</td>
<td>2.029</td>
<td>0.120</td>
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<tr>
<td>EcIspG reduced</td>
<td>2.040</td>
<td>1.900</td>
<td>1.900</td>
<td>1.950</td>
<td>0.140</td>
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<tr>
<td>PaIspG reduced</td>
<td>2.032</td>
<td>1.901</td>
<td>1.899</td>
<td>1.944</td>
<td>0.133</td>
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<tr>
<td>EcIspG + 7</td>
<td>2.022</td>
<td>1.875</td>
<td>1.870</td>
<td>1.922</td>
<td>0.152</td>
</tr>
<tr>
<td>PaIspG + 7</td>
<td>2.029</td>
<td>1.892</td>
<td>1.848</td>
<td>1.923</td>
<td>0.182</td>
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<tr>
<td>EcIspG “X”</td>
<td>2.092</td>
<td>2.018</td>
<td>1.999</td>
<td>2.036</td>
<td>0.093</td>
</tr>
<tr>
<td>TtIspG “X”</td>
<td>2.070</td>
<td>2.019</td>
<td>2.000</td>
<td>2.035</td>
<td>0.087</td>
</tr>
<tr>
<td>AtIspG “X”</td>
<td>2.097</td>
<td>2.019</td>
<td>2.000</td>
<td>2.039</td>
<td>0.097</td>
</tr>
<tr>
<td>EcIspG + 5</td>
<td>2.087</td>
<td>2.012</td>
<td>2.003</td>
<td>2.034</td>
<td>0.085</td>
</tr>
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</table>

$^a g_{iso} = (g_1 + g_2 + g_3) / 3$, $^b \Delta g = g_3 - g_1$.

Table S2. IC$_{50}$s for EcIspG, PaIspG, and AtIspG.

<table>
<thead>
<tr>
<th>Enzyme + Inhibitor</th>
<th>IC$_{50}$ (µM)</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec IspG + 7</td>
<td>2.5</td>
<td>0.994</td>
</tr>
<tr>
<td>Ec IspG + 8</td>
<td>1.4</td>
<td>0.988</td>
</tr>
<tr>
<td>Pa IspG + 7</td>
<td>2.4</td>
<td>0.985</td>
</tr>
<tr>
<td>Pa IspG + 8</td>
<td>0.80</td>
<td>0.995</td>
</tr>
<tr>
<td>At IspG + 7</td>
<td>1.7</td>
<td>0.966</td>
</tr>
<tr>
<td>At IspG + 8</td>
<td>2.0</td>
<td>0.982</td>
</tr>
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Table S3. Hyperfine tensors determined from ENDOR and HYSCORE simulations of labeled substrates, 2 and 5, binding to IspG, and distances estimates. The uncertainties of distance estimates are based on an assumed uncertainty of 0.5 MHz for the tensors.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>$A_{11}$ (MHz)</th>
<th>$A_{22}$ (MHz)</th>
<th>$A_{33}$ (MHz)</th>
<th>$A_{iso}$ (MHz)</th>
<th>$\alpha$ (°)</th>
<th>$\beta$ (°)</th>
<th>$\gamma$ (°)</th>
<th>$r$ ($K_{min}$) (Å)</th>
<th>$r$ ($K_{max}$) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-$^{13}$C] -2</td>
<td>-0.7</td>
<td>-0.9</td>
<td>2.2</td>
<td>0.2</td>
<td>-30</td>
<td>0</td>
<td>30</td>
<td>2.6 ± 0.4</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>[2-$^{13}$C] -2</td>
<td>0.5</td>
<td>0.7</td>
<td>4.9</td>
<td>2.0</td>
<td>36</td>
<td>15</td>
<td>9</td>
<td>2.3 ± 0.3</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>[3-$^{13}$C] -2</td>
<td>3.9</td>
<td>-0.3</td>
<td>-0.1</td>
<td>1.2</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>2.3 ± 0.3</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>[3-$^{13}$C] -5</td>
<td>4.5</td>
<td>3.0</td>
<td>10.0</td>
<td>5.8</td>
<td>80</td>
<td>30</td>
<td>60</td>
<td>2.0 ± 0.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>[4-$^{13}$C] -5</td>
<td>5.4</td>
<td>1.5</td>
<td>3.8</td>
<td>3.6</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>2.6 ± 0.4</td>
<td>3.2 ± 0.5</td>
</tr>
</tbody>
</table>
Figure S1. Amino-HMBPP (7) + PflspH HYSCORE spectra. (A) 3420 G, $\tau = 136$ ns. (B) 3420 G, $\tau = 200$ ns. $T = 10$ K.
Figure S2. Experimental and simulated HYSCORE spectra of amino-HMBPP (7) + EclspH.
Experimental condition: $B_0 = 3440$ G, $\nu = 9.665$ GHz with (A) $\tau = 136$ ns. (B) $\tau = 172$ ns. (C) $\tau = 208$ ns. (D) $\tau = 244$ ns. Simulation parameters: $A_i = [1.85, 1.25, 3.70]$ MHz, $\alpha = 0^\circ$, $\beta = 22^\circ$, $\gamma = 20^\circ$, $e^2 qQ/h = 0.8$ MHz, $\eta = 0.2$. 
Figure S3. 9.05 GHz CW-EPR and UV-Vis spectra of AaIspH + 8. (A) CW-EPR spectra of 1.3 mM AaIspH + 20 eq dithionite (blue) and 1.3 mM AaIspH + 20 eq dithionite + 10 eq 8 (red). $T = 10K$, power = 2 mW. Inset: CW-EPR spectra of the same two samples with extended magnetic field range (500 G – 5500 G). (B) UV-Vis spectra of AaIspH oxidized (blue); reduced (green); + 8 (cyan); + 8 + dithionite (red). 0.05mM AaIspH, 50 eq dithionite, 50 eq 8. UV-Vis spectra taken in an anerobic environment ($O_2 < 2$ ppm) at 25 °C.
Figure S4. Enzyme activity inhibition curves for EcIspG (A,B), PaIspG (C,D), and AtIspG (E,F) with 7 and 8.
Figure S5. [1-17O]-HMBPP + PalspG HYSCORE spectra. (A) 3315 G, \( \tau = 136 \) ns. (B) 3315 G, \( \tau = 200 \) ns. (C) 3435 G, \( \tau = 136 \) ns. (D) 3435 G, \( \tau = 200 \) ns. (E) 3480 G, \( \tau = 136 \) ns. (F) 3480 G, \( \tau = 200 \) ns. \( T = 10 \) K.
Figure S6. [U-\textsuperscript{13}C]-HMBPP + \textit{P}aIspG HYSCORE spectra (top) and \textsuperscript{13}C simulations (below) zoomed in to [-10 10 0 10] MHz ([xmin xmax ymin ymax]). (A) 3315 G, $\tau$ = 136 ns. (B) 3435 G, $\tau$ = 136 ns. (C) 3480 G, $\tau$ = 136 ns. (D) 3315 G, $\tau$ = 200 ns. (E) 3435 G, $\tau$ = 200 ns. (F) 3480 G, $\tau$ = 200 ns. $T$ = 10 K. (3-\textsuperscript{13}C) $A_{ii}$ = [3.9 -0.3 -0.1] MHz. (3-\textsuperscript{13}C) [$\alpha \beta \gamma$] = [0 20 0] degrees. (2-\textsuperscript{13}C) $A_{ii}$ = [0.5 0.7 4.9] MHz. (2-\textsuperscript{13}C) [$\alpha \beta \gamma$] = [36 15 9] degrees. (1-\textsuperscript{13}C) $A_{ii}$ = [-0.7 -0.9 2.2] MHz. (1-\textsuperscript{13}C) [$\alpha \beta \gamma$] = [-30 0 30] degrees.
Figure S7. [1-13C]-HMBPP + PaIspG HYSCORE spectra (top) and 13C simulations (below) zoomed in to [-10 10 0 10] MHz ([xmin xmax ymin ymax]). (A) 3315 G, \( \tau = 136 \) ns. (B) 3435 G, \( \tau = 136 \) ns. (C) 3480 G, \( \tau = 136 \) ns. (D) 3315 G, \( \tau = 200 \) ns. (E) 3435 G, \( \tau = 200 \) ns. (F) 3480 G, \( \tau = 200 \) ns. \( T = 10K \). \( A_{ii} = [-0.7 -0.9 2.2] \) MHz. \( \alpha = -30^\circ \). \( \beta = 0^\circ \). \( \gamma = 30^\circ \).
Figure S8. [2-\textsuperscript{13}C]-HMBPP + PaIspG HYSCORE spectra (top) and \textsuperscript{13}C simulations (below) zoomed in to [-10 10 0 10] MHz ([xmin xmax ymin ymax]). (A) 3315 G, $\tau = 136$ ns. (B) 3435 G, $\tau = 136$ ns. (C) 3480 G, $\tau = 136$ ns. (D) 3315 G, $\tau = 200$ ns. (E) 3435 G, $\tau = 200$ ns. (F) 3480 G, $\tau = 200$ ns. $T = 10$ K. $A_{ii} = [0.5 0.7 4.9]$ MHz. $\alpha = 36^\circ$. $\beta = 15^\circ$. $\gamma = 9^\circ$. 

A

B

C

D

E

F

S23
Figure S9. [3-^{13}C]-HMBPP + PaIspG HYSCORE spectra (top) and $^{13}$C simulations (below) zoomed in to [-10 10 0 10] MHz ([xmin xmax ymin ymax]). (A) 3315 G, $\tau = 136$ ns. (B) 3435 G, $\tau = 136$ ns. (C) 3480 G, $\tau = 136$ ns. (D) 3315 G, $\tau = 200$ ns. (E) 3435 G, $\tau = 200$ ns. (F) 3480 G, $\tau = 200$ ns. $T = 10K$. $A_{ij} = [3.9 -0.3 -0.1]$ MHz. $\alpha = \gamma = 0^\circ$. $\beta = 20^\circ$. 

A

B

C

D

E

F
Figure S10. 9.05 GHz CW-EPR freeze-quench spectra of IspG. (A) 500 μM EcIspG + 80 eq dithionite + 20 eq 7, incubated for 10 minutes. (B) 600 μM PaIspG* + 80 eq dithionite with no inhibitor added. (C,D) 600 μM PaIspG + 80 eq dithionite + 20 eq 7, incubated 30 seconds (C) and 600 seconds (D). Red arrows indicate signal due to bound 2, displaced later (as seen in D) by 7. Simulation shown in green. T = 8 K, Power = 5 mW.
Figure S11. *PaIspG + 7* HYSCORE spectra.  (A) magnetic field = 3650 G, $\tau = 136$ ns, $T = 6$ K.  (B) pH = 8.6, magnetic field = 3660 G, $\tau = 140$ ns, $T = 7$ K.  (C) pH = 8.6, magnetic field = 3430 G, $\tau = 140$ ns, $T = 7$ K.
Figure S12. 9.05 GHz CW-EPR temperature dependence spectra of PalspG with 2 or 7. (A) 600 μM PalspG + 40 eq dithionite + 20 eq 2 at 10 (blue) and 20 K (red), incubated for 10 min. (B) 600 μM PalspG + 80 eq dithionite + 20 eq 7 at 8 (blue) and 20 K (red), incubated for 10 min. All spectra acquired at power = 5 mW.
Figure S13. CW-EPR spectra of *T* *t*IspG D87A. Microwave frequency $\sim$ 9 GHz. Power = 5-10 mW. $T \sim$ 8 K. (A) (reduced, “-L”) 0.9 mM *T* *t*IspG D87A + 40 eq dithionite. (B) 1 mM *T* *t*IspG D87A + 20 eq amino-HMBPP (7) + 30 eq dithionite. (C) 0.9 mM *T* *t*IspG D87A + 20 eq HMBPP (2) + 30 eq dithionite.
References


